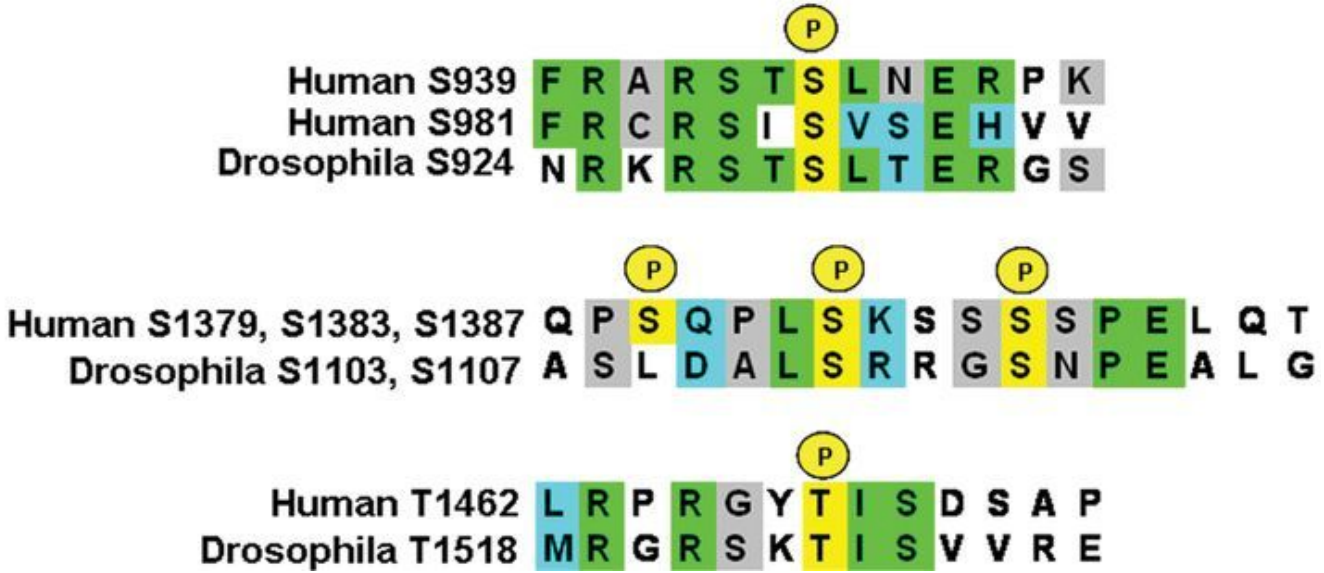
Phosphoproteomics

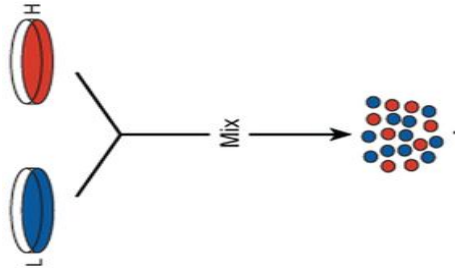


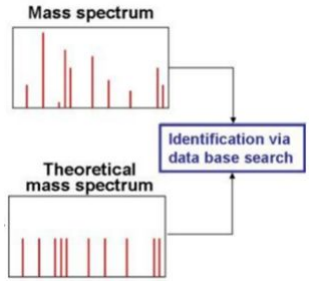
Jacob Sykalski & Emanuel Perez

Preview

Phosphorylation and phosphoproteomics

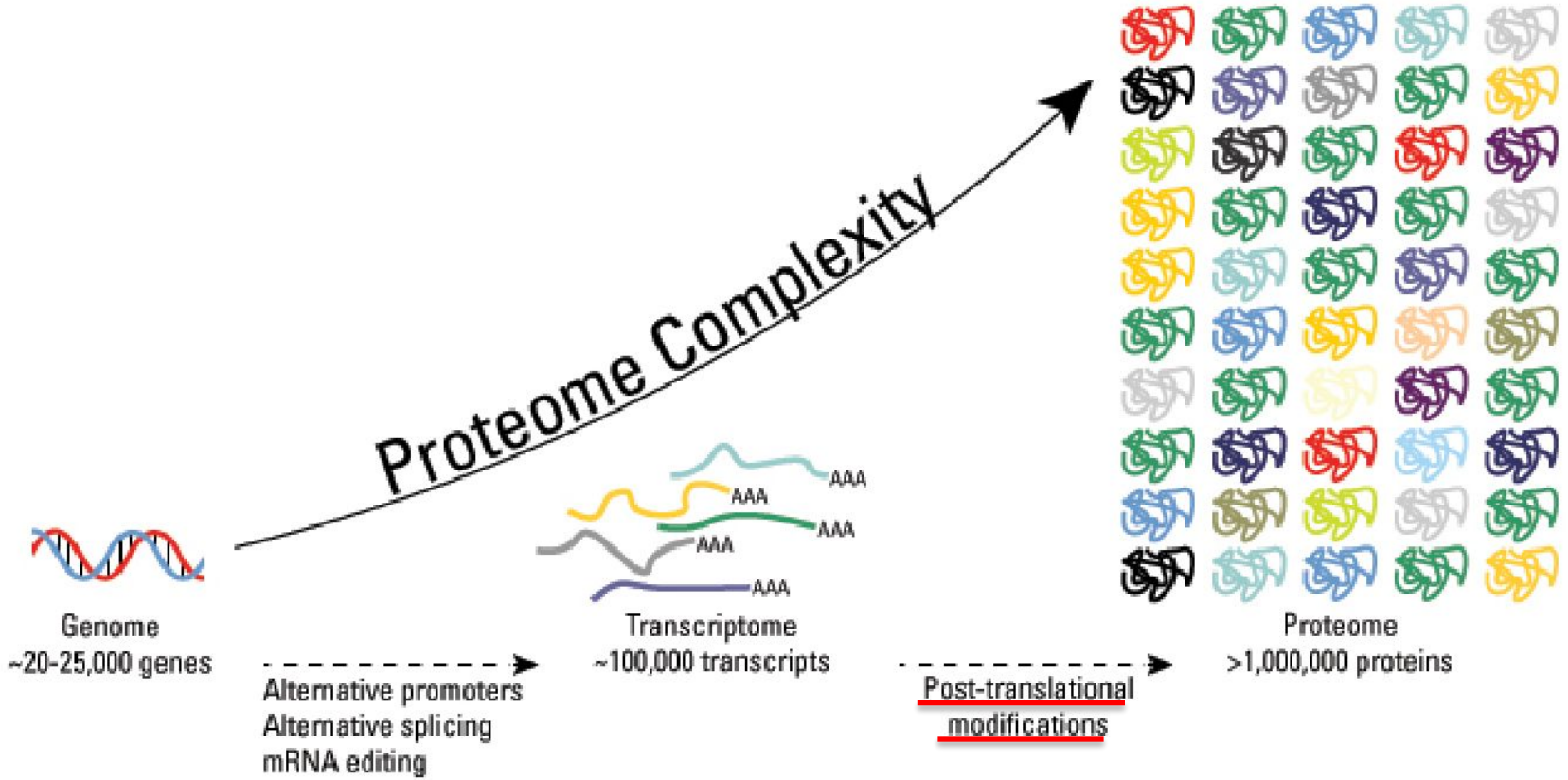
Mapping and identifying phosphorylation sites



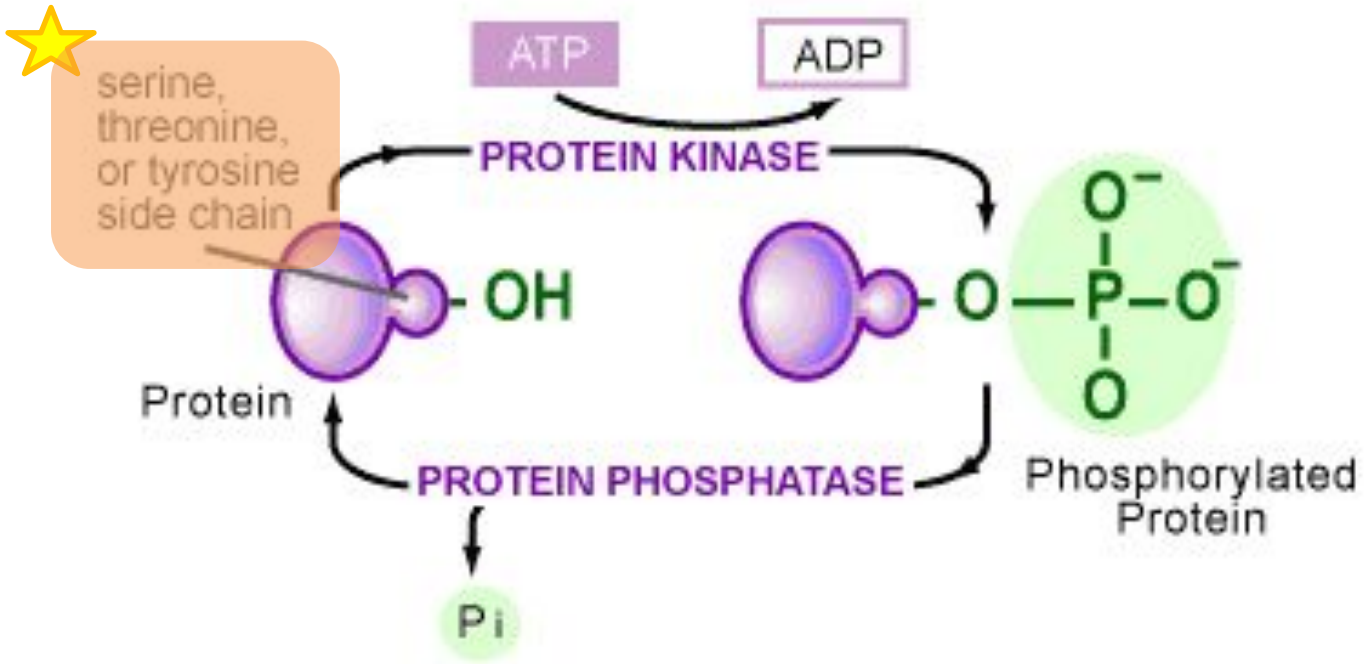


SILAC

How does the *proteome* become complex?

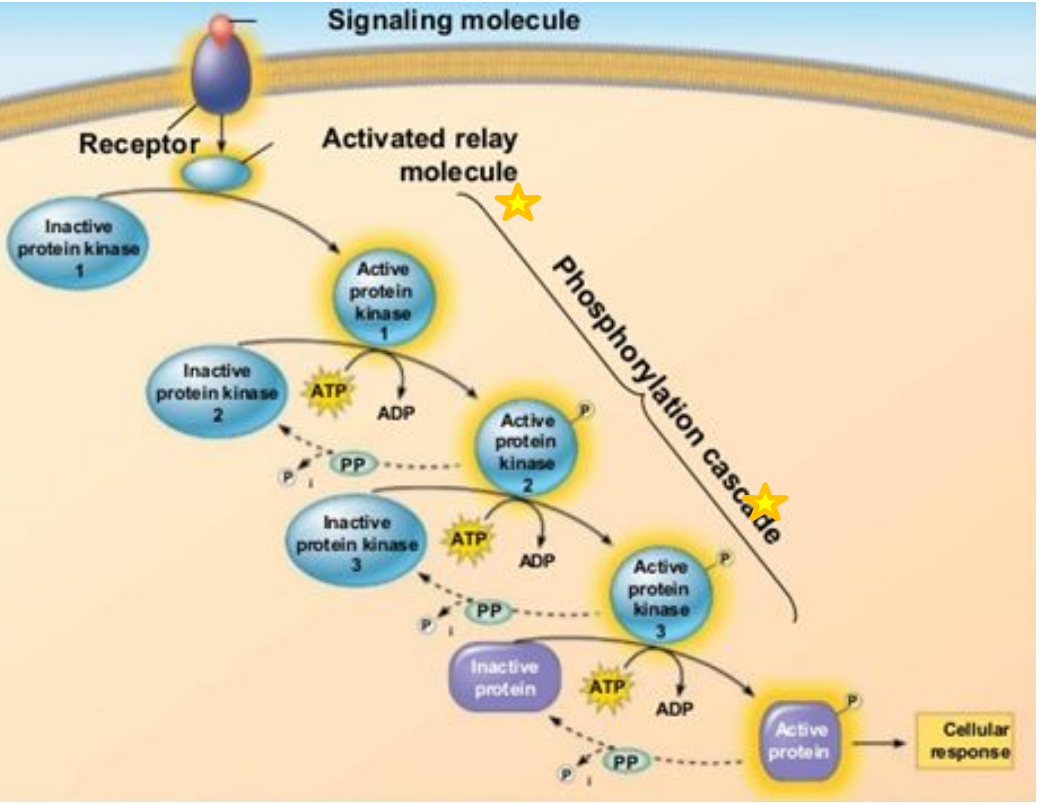


What is *phosphorylation*?



The addition of a phosphate group onto a protein by a kinase

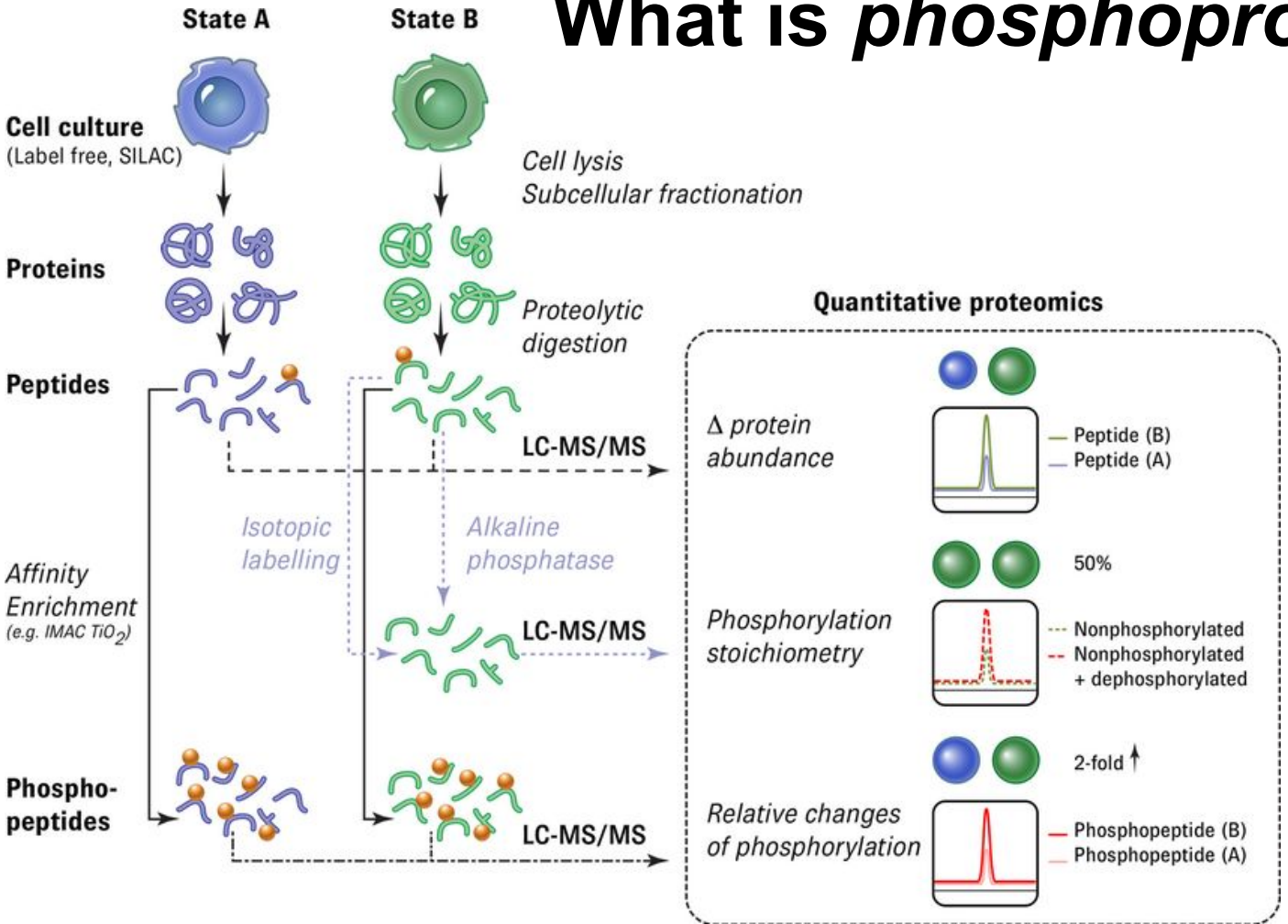
Why is *phosphorylation important*?



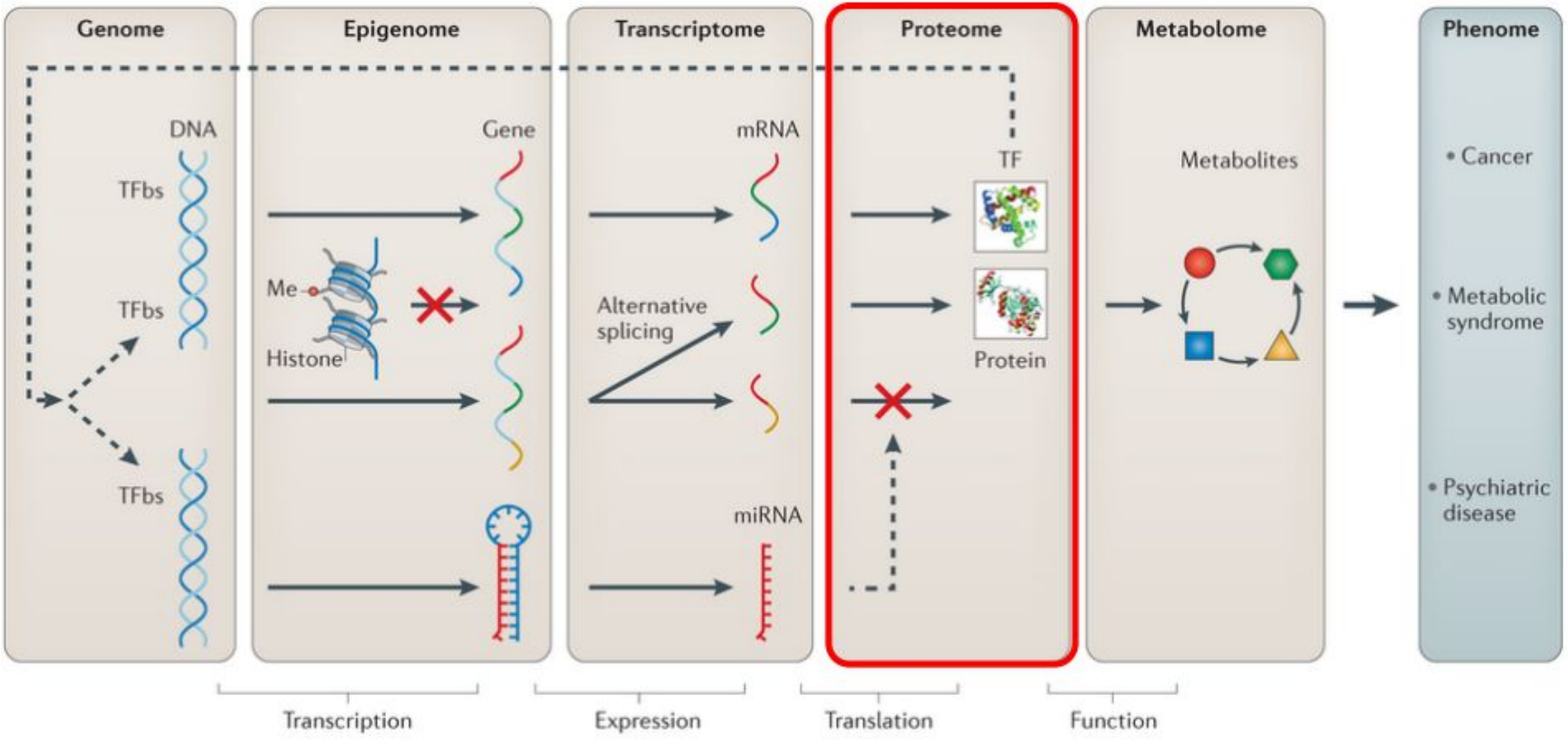
Regulation of cell signaling pathways

(Phosphorylation cascades)

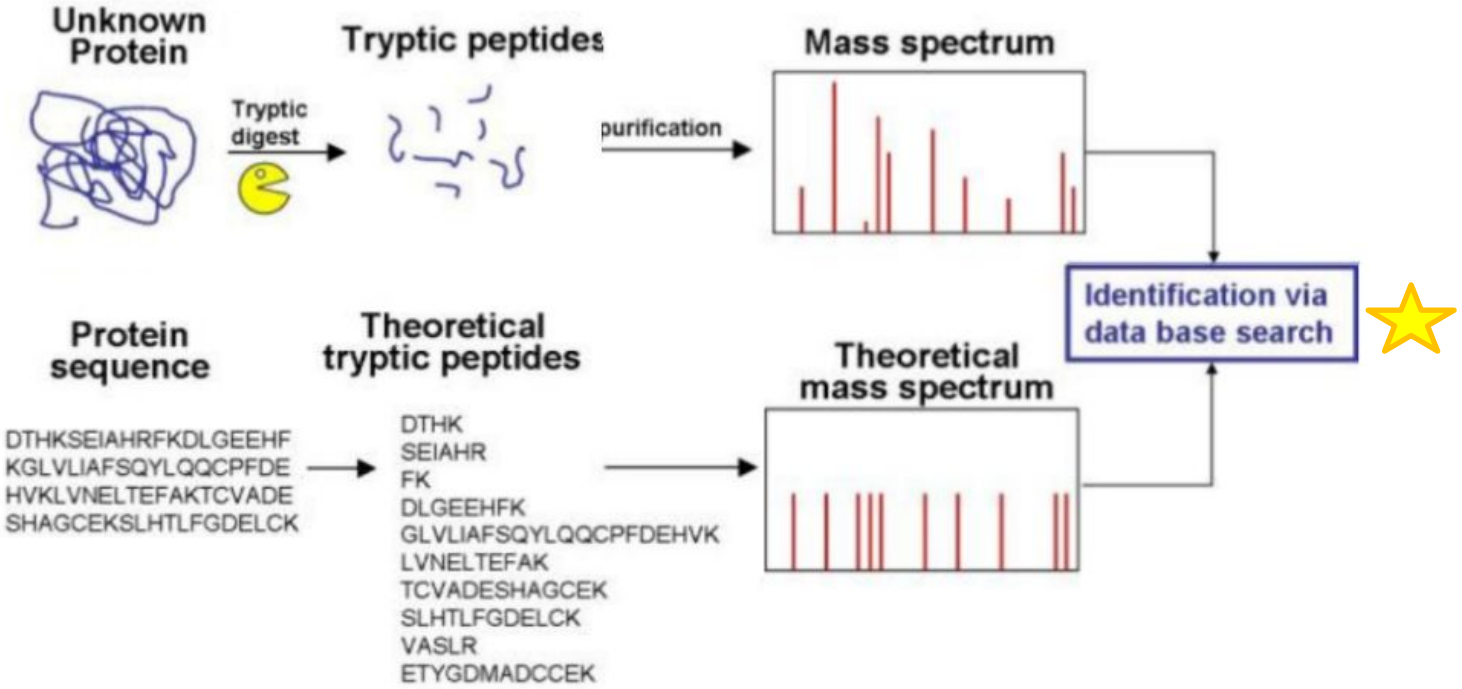
What is *phosphoproteomics*?



Why is (quantitative) *phosphoproteomics important*?

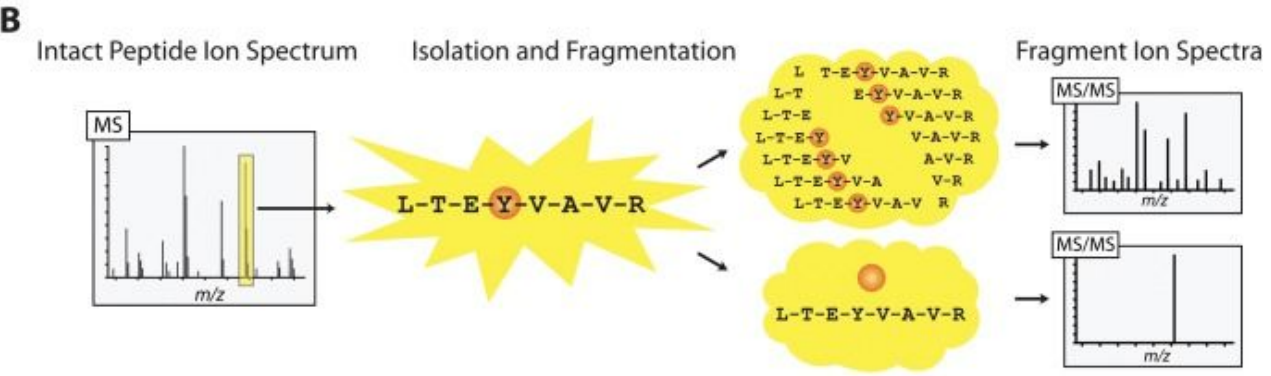


How do we *identify proteins*?

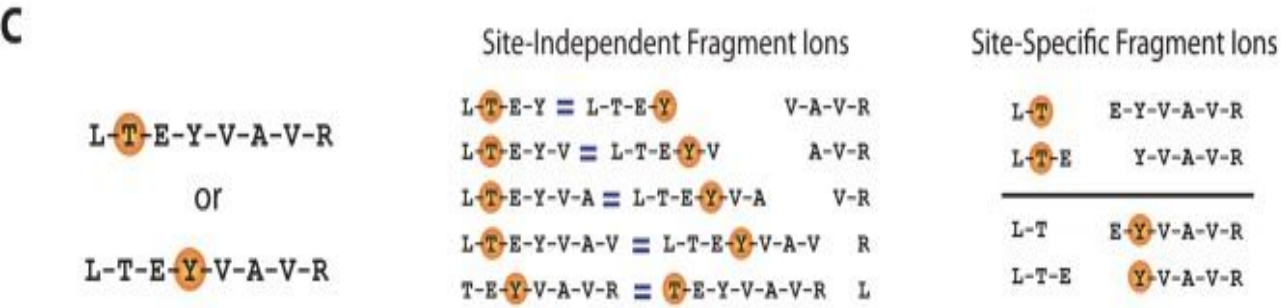


Mass spectrometry

What information is derived from MS?



- Peptide ion **masses**
- Peptide **positions**



What does this type of mass spectrometer look like?

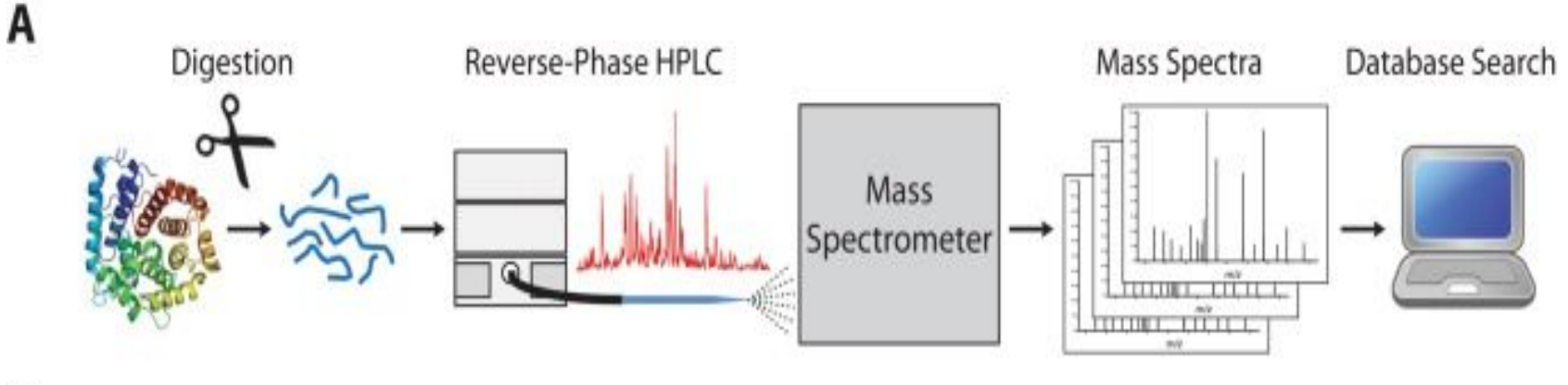
~4.6 feet tall

New advances of MS allow rapid identification of phosphorylation sites with precision and sensitivity

How do you map phosphorylation sites?

Peptides separated

Peptide matching



Protein samples digested with a proteolytic enzyme

Peptides enter the mass spectrometer

Issues complicating phosphopeptide identification?

Phosphate moiety is susceptible to being broken down

Scarcity of phosphorylation within the protein of interest

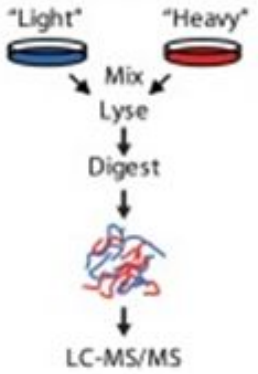
Not always possible to identify the precise site of modification

Reliance on a single sequence-specific protease

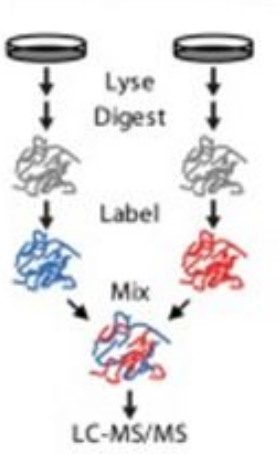
And more!

What are the labeling methods?

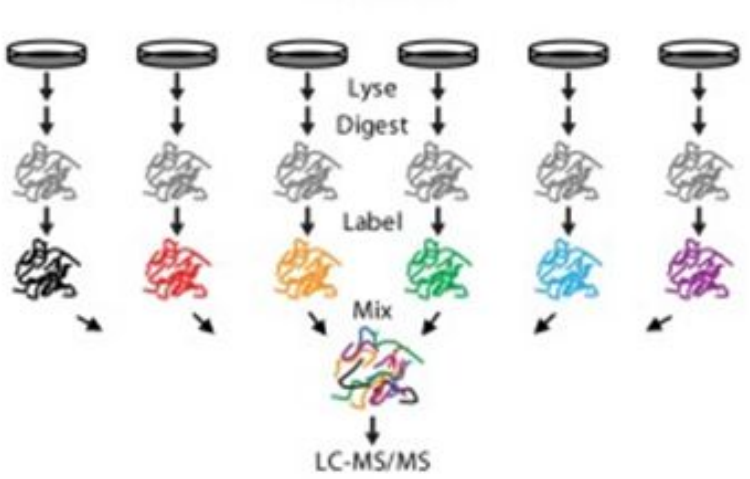
Metabolic Labels



Chemical Labeling



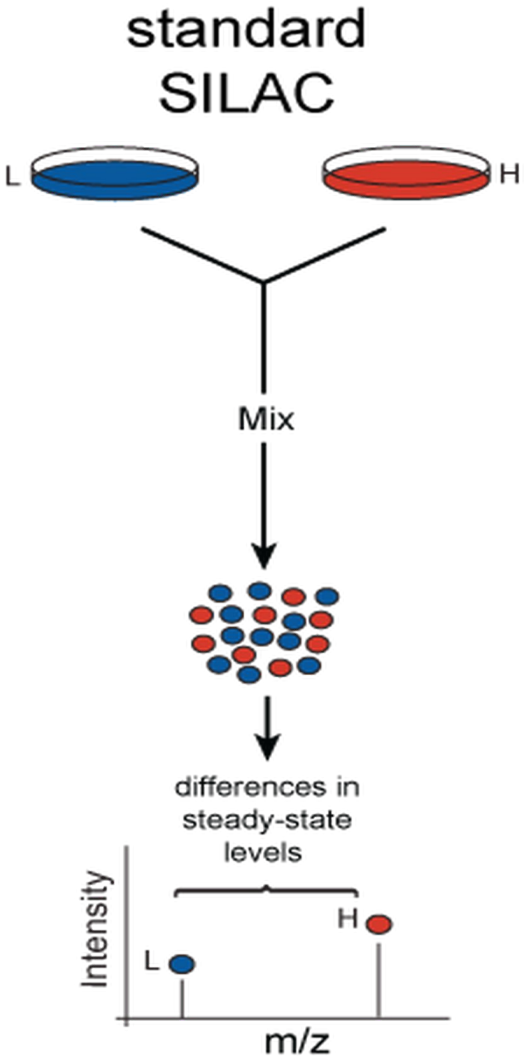
Isobaric Labels



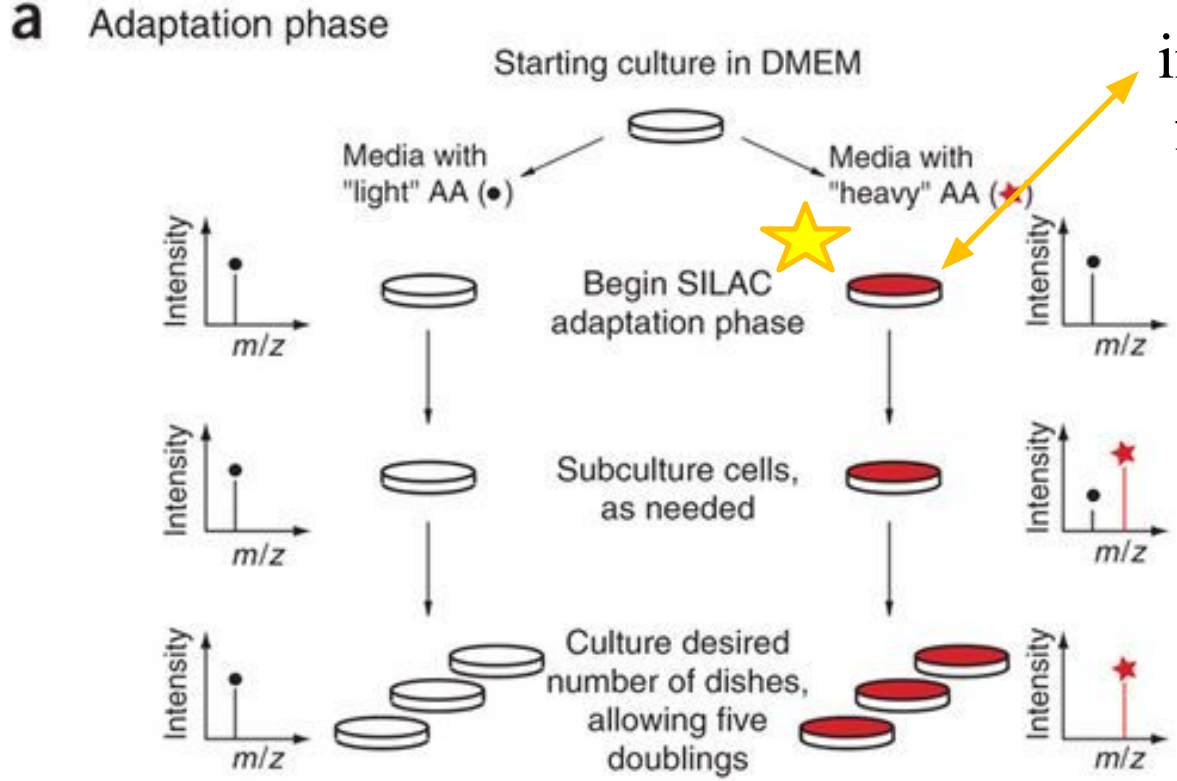
What is *SILAC*?

Stable **I**sotope **L**abeling with **A**mino acids in **C**ell culture

the most popular *metabolic* labeling method

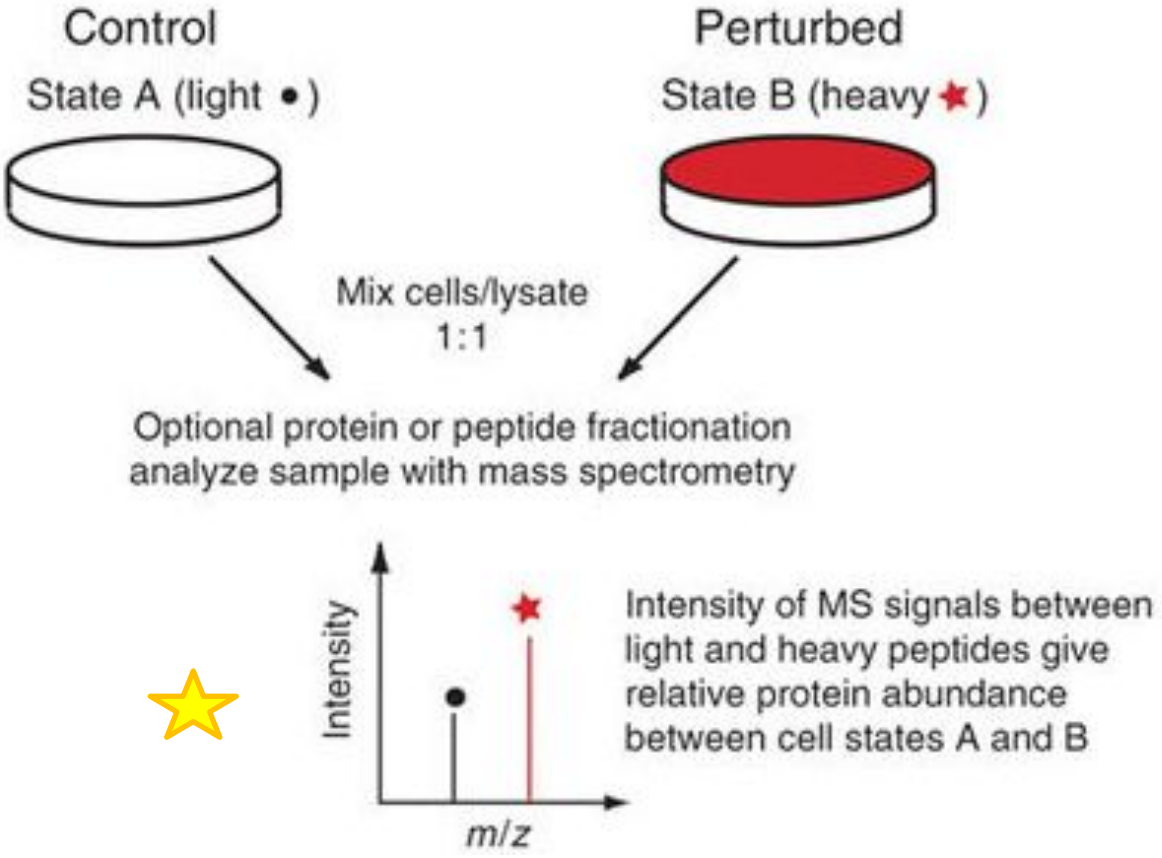


How are SILAC cell pools distinguishable by MS?



introduce a mass difference for relative quantification

b Experiment phase



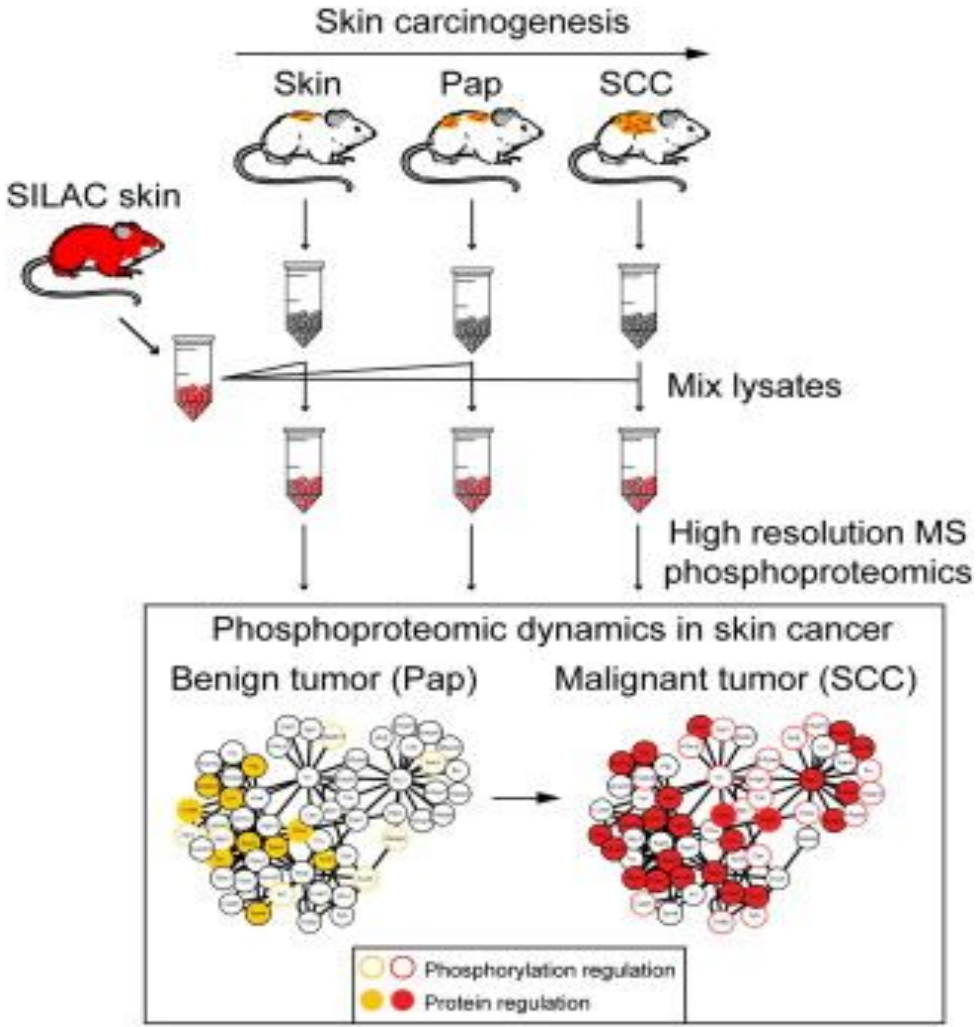
Advantages vs. *disadvantages* of SILAC

Accurate relative quantification
No need for chemical derivatization or manipulation
Purification step does not affect relative concentrations
Data analysis is easier
Adapted to almost any cell system

Challenging to perform in vivo
Protein loss due to mass spec

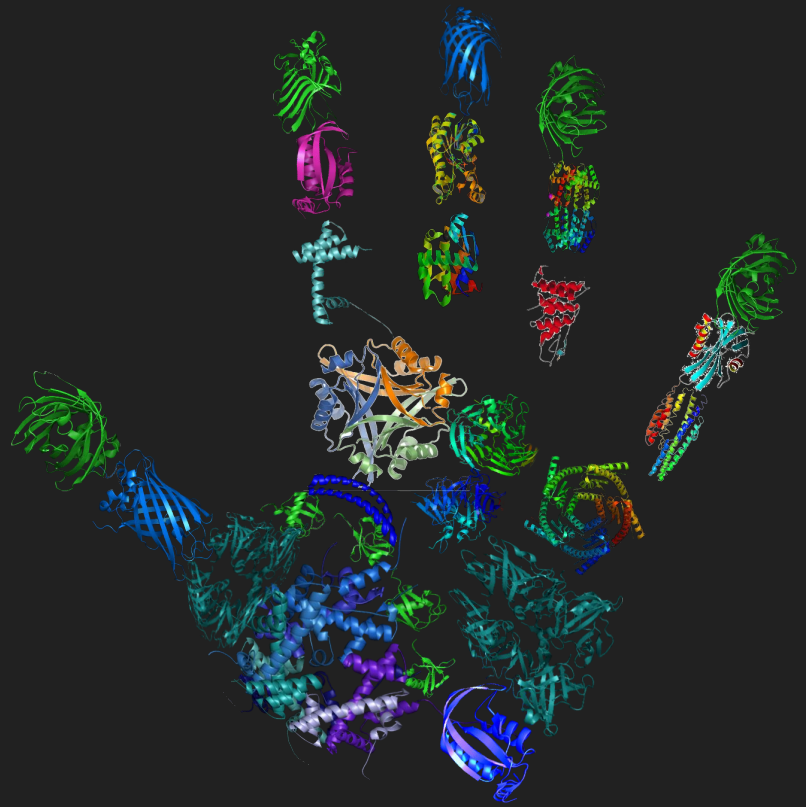
Why are SILAC labeled mice useful?

Why is phosphoproteomics useful to study *cancer*?

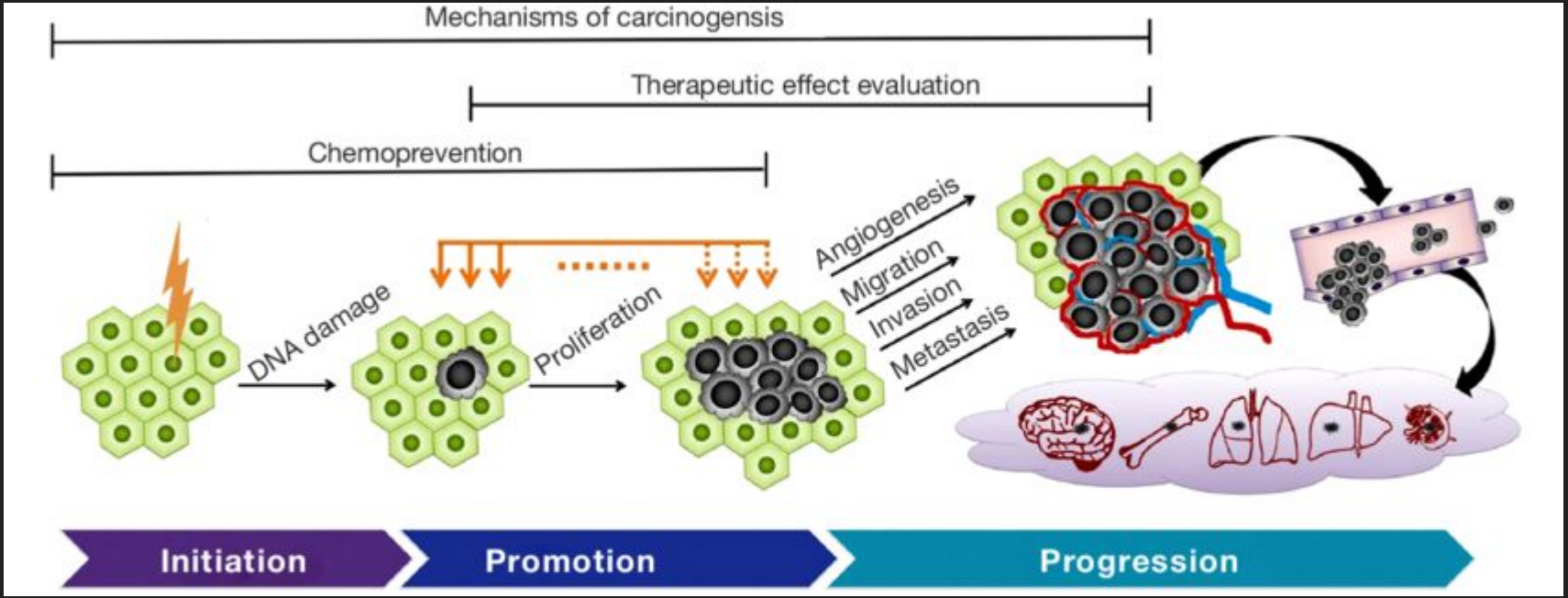


In Vivo SILAC-Based Proteomics Reveals Phosphoproteome Changes during Mouse Skin Carcinogenesis

Zanivan et al 2013 Cell Rep. 2013 Feb 21



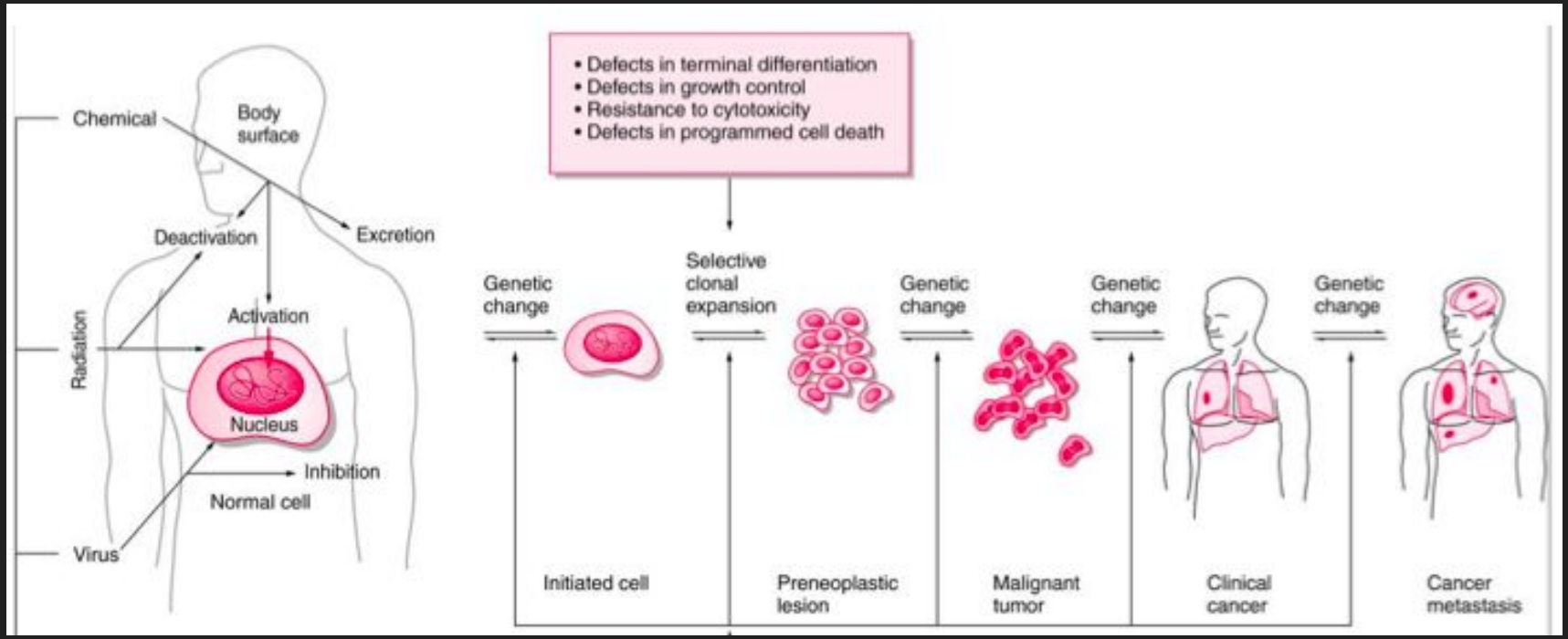
What is carcinogenesis?



Transformation of normal cells to cancer cells through cellular, genetic and epigenetic changes to cellular cell division

Tomasetti C, Li L, Vogelstein B (23 March 2017)

What are the stages?



Benign: not cancerous, they respond well to treatment & unable to spread

Malignant: able to metastasize, grow quickly, and can invade other

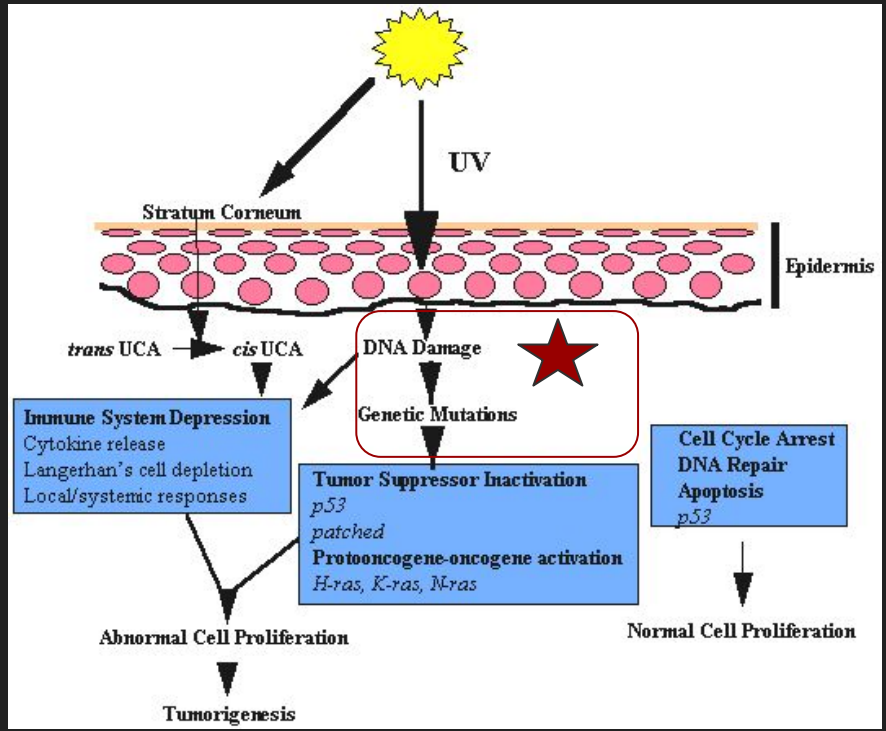
Cynthia Dennison Haines MD, on April 1, 2005

How does UV light initiate cancer?

Activation of proto oncogenes

Inactivation of tumor suppressor genes

Inactivation of genomic stability genes



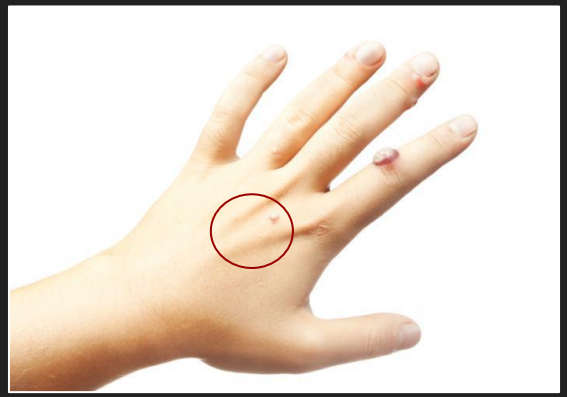
Differences between PAP and SCC tumors?

Papilloma(Pap)

Benign epithelial growing tumor

Grows slower

Spherical shaped outgrowth

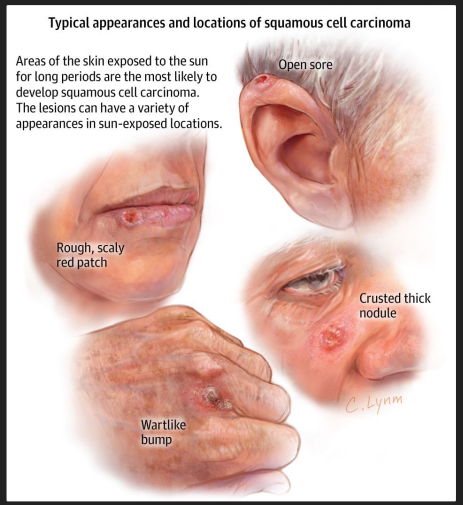


Squamous Cell Carcinoma (SCC)

SCC are the thin, flat cells considered cancerous and appear in late onset

Grows aggressively

Scaly red patches, open sores, war like,
or thick nodules



Why mouse models？

Mouse Models

Long history and supporting infrastructure

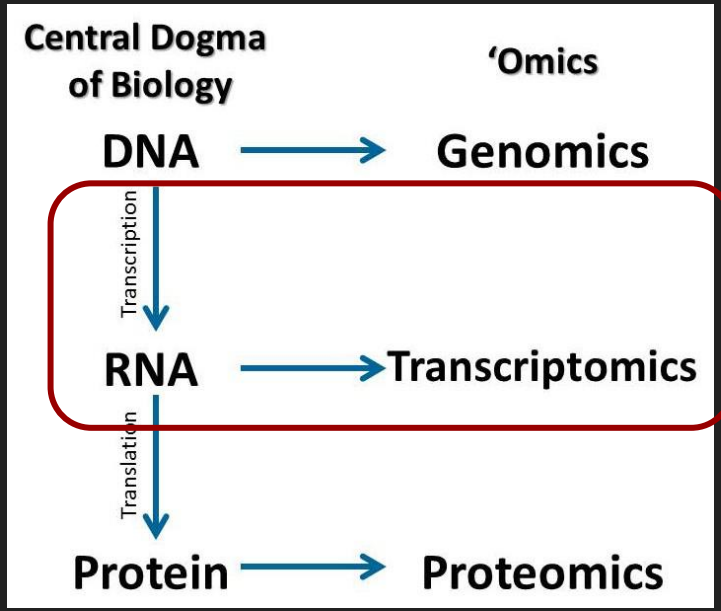
Complex disease can be easily manipulated

Highcross-species similarity with humans


CHALLENGES

Challenges

Transcriptomics and gene expression heavily use mRNA expression levels

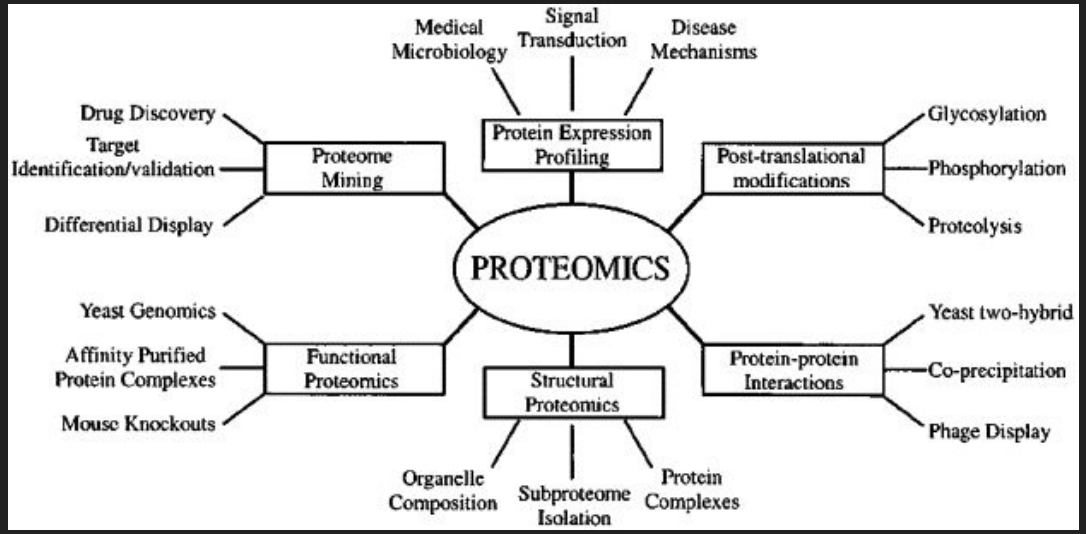


mRNA levels do not correlate well with protein expression levels

Challenges

Quantitative proteomic studies

in vivo is difficult



No quantitative in-depth studies of the phosphoproteome have been conducted in vivo for cancer

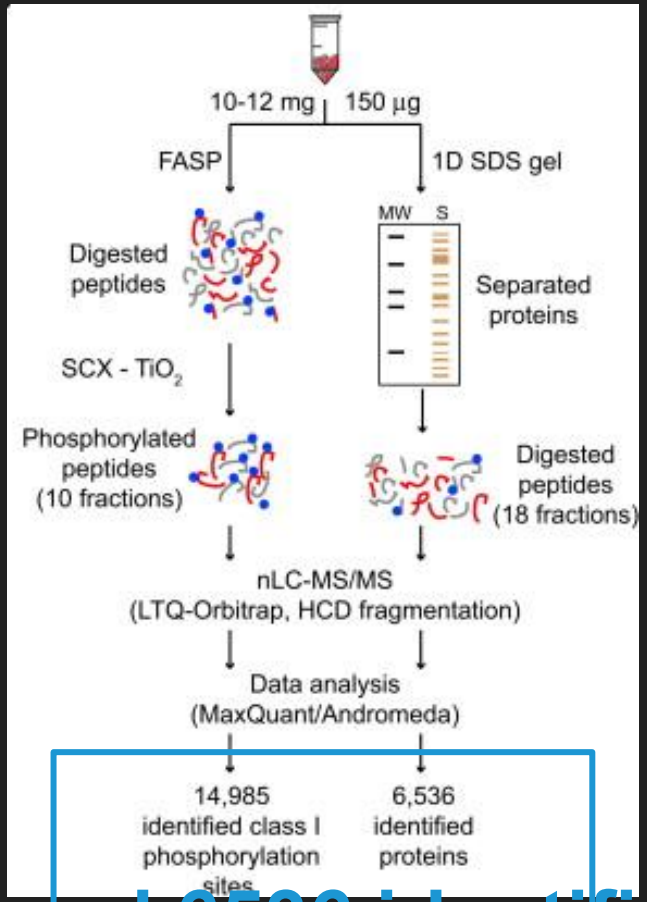
How were proteins and phosphorylated sites identified?

1) SILAC Lysate mixture is separated by SDS-PAGE

2) SCX-TIO2 chromatography used to fractionate peptides

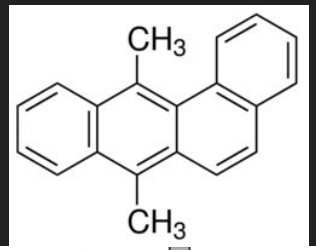
3) Filter aided sample preparation

4) Liquid Chromatography and Orbitrap MS to analyze peptide fractions
5) MaxQuant/ Andromeda data analysis



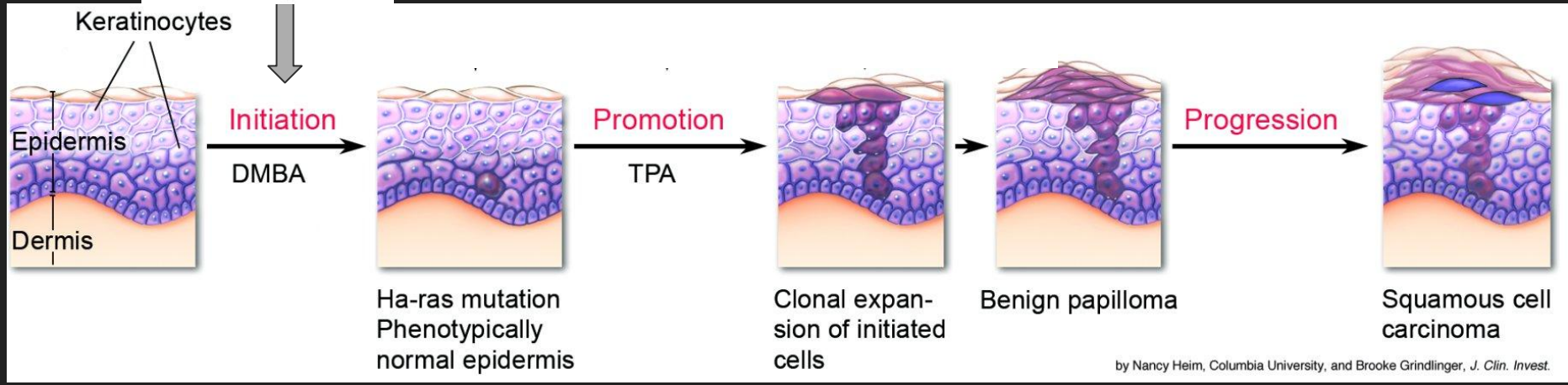
14, 985 class 1 phosp. sites and 6536 identified proteins identified

How did they induce carcinogenesis?

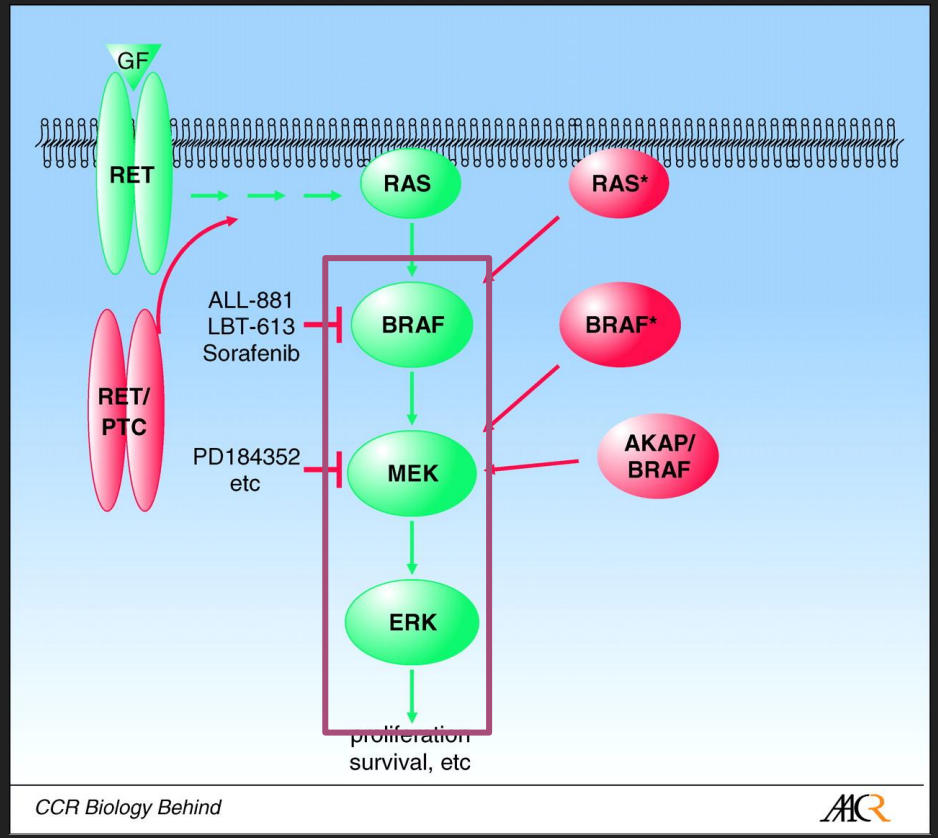


7,12-Dimethylbenz(a)anthracene

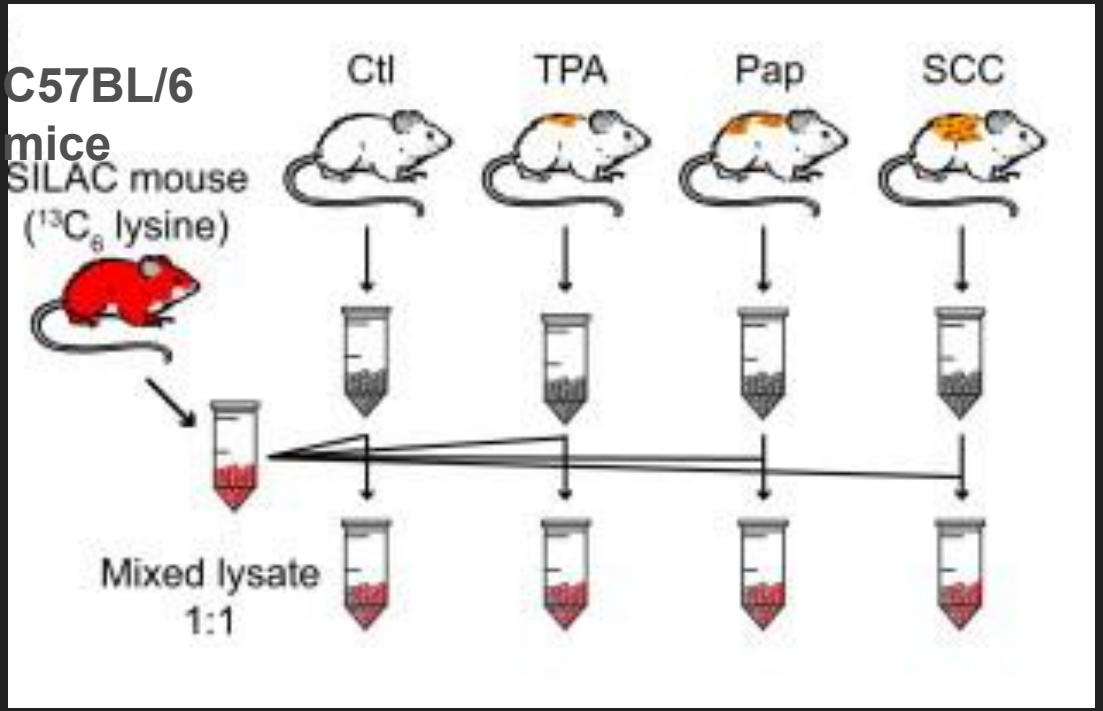
Papilloma



How is the Hras pathway involved in tumor growth



How did they isolate cell tissue?



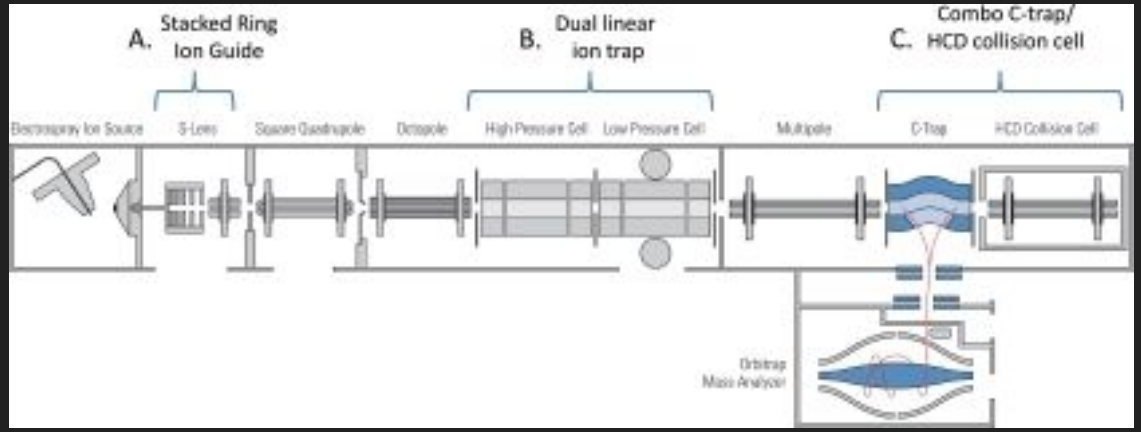
Lysed in 4% SDS, 100 mM DTT, 100 mM Tris HCl lysis buffer. Mixed 1:1 with SILAC skin lysate

Pooled tissues collected from 3-6 different mice

>95% of the quantified proteins and phosphorylation sites in the skin, Ctl or TPA, were within a 4-fold ratio compared to the SILAC skin.

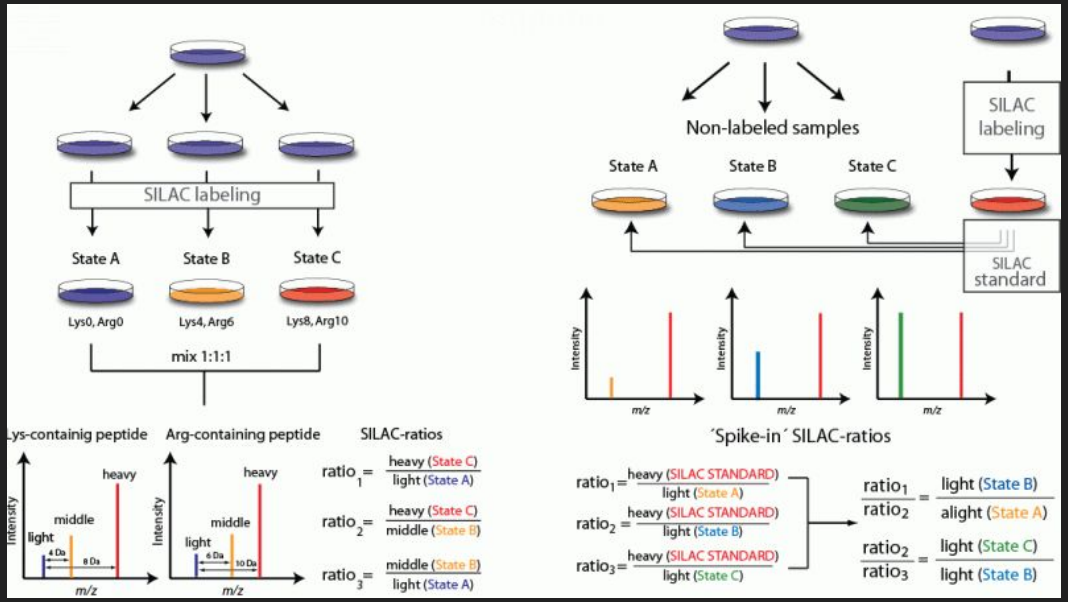
How were peptides quantified?

LTQ-Orbitrap Velos



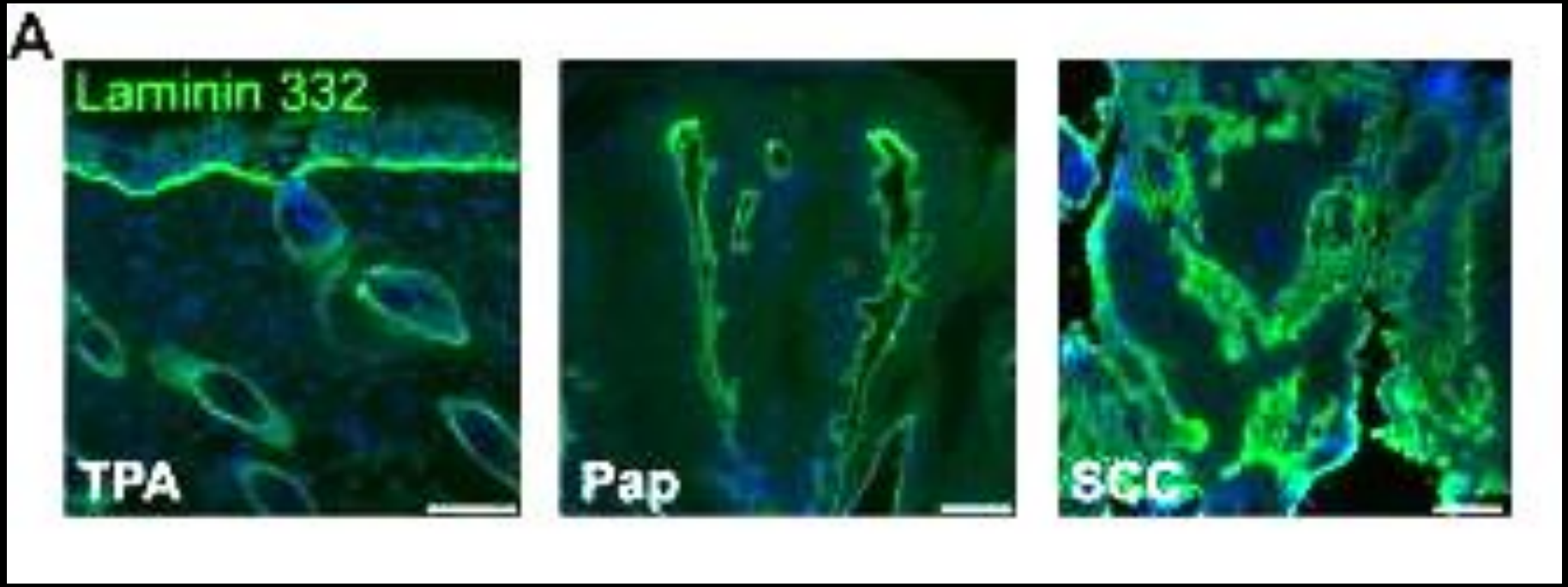
http://planetorbitrap.com/orbitrap-elite#.WsO6z_Dwbrc

How does “Spike in” SILAC differ?



“Spike-in” standard solves the labeling problem because the quantification of each of the tissue samples can be performed relative to a standard.

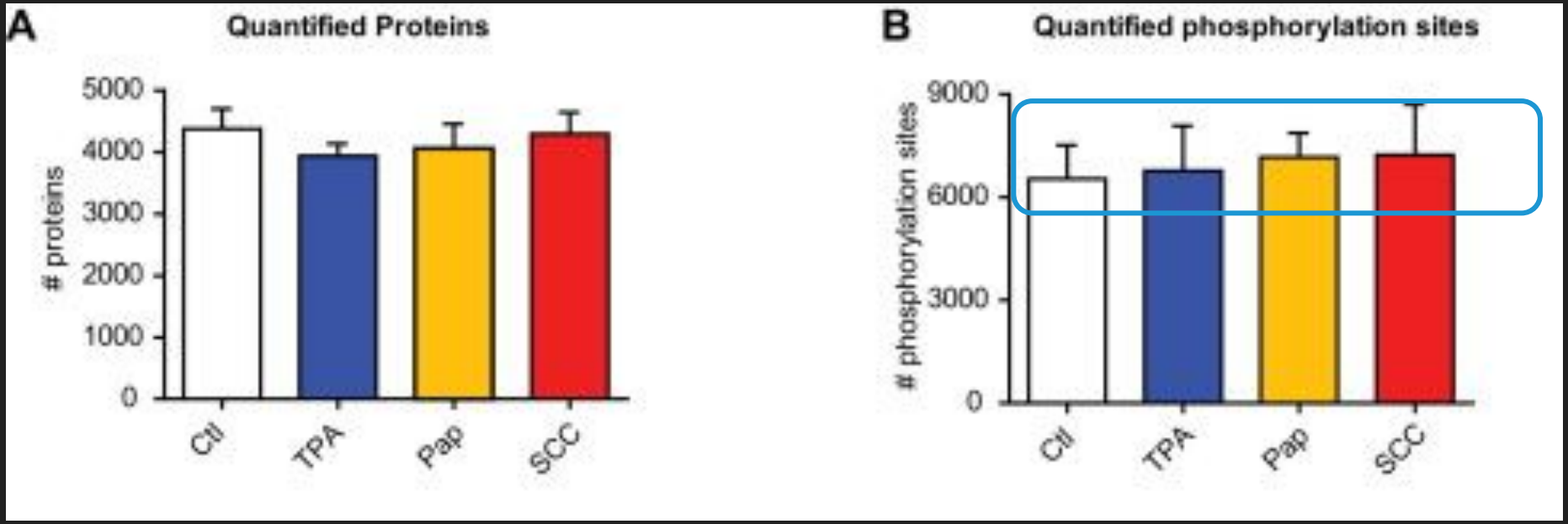
How were cancer stage classifications identified?



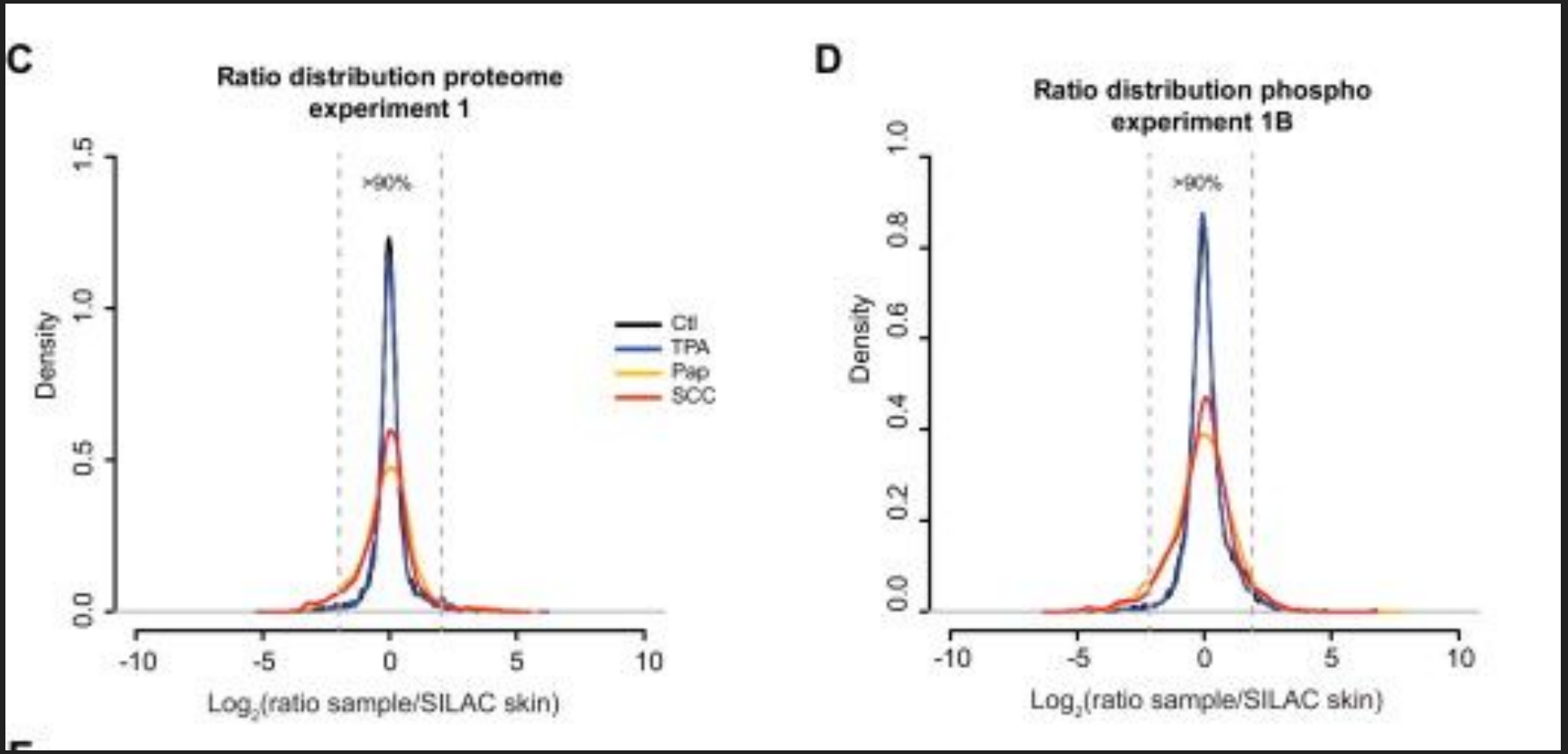
(A) Confocal images of frozen sections of TPA, Pap, and SCC stained for laminin 332

Laminin 332 receptor integrin β1 was upregulated specifically in SCC as most of the proteins of the cell adhesion subnetwork. Intriguingly, most of these proteins, including Fscn1, are functionally and physically connected to the actin cytoskeleton that is a critical regulator of cancer cell motility and invasion.

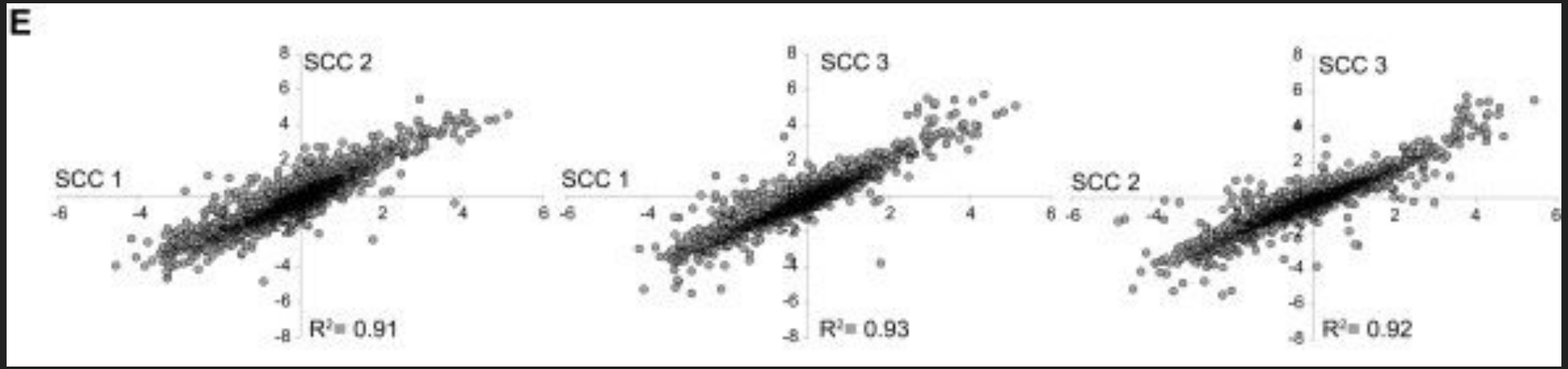
How did the protein and phosphorylation sites differ among groups?



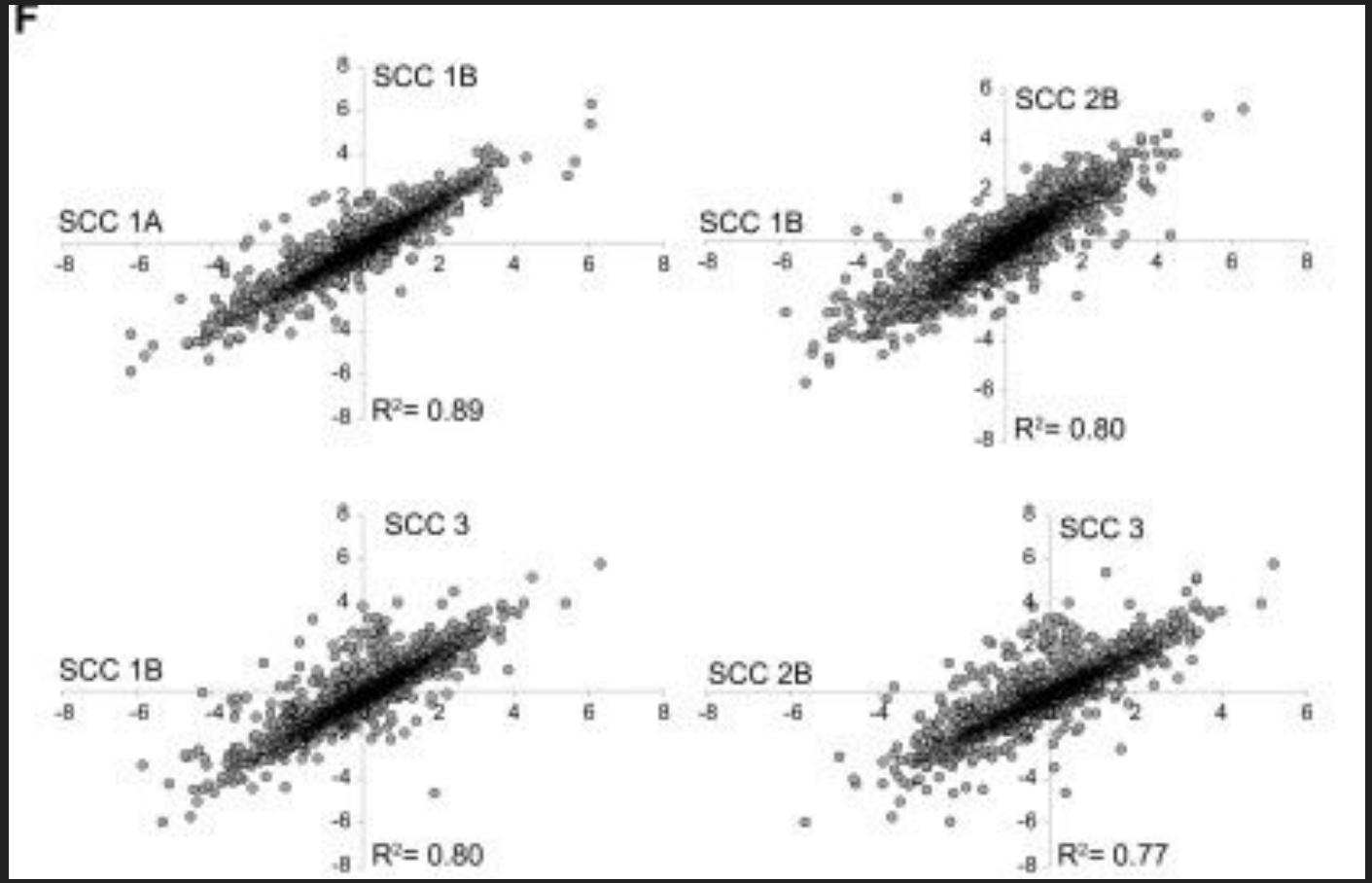
Increased number of phosphorylation sites found in each tissue sample vs control showing an increasing trend with later stages of skin carcinoma



>95% of proteins and phosphorylation sites were within a 4 fold ratio compared to the SILAC skin. Validate SILAC as great spike in standard

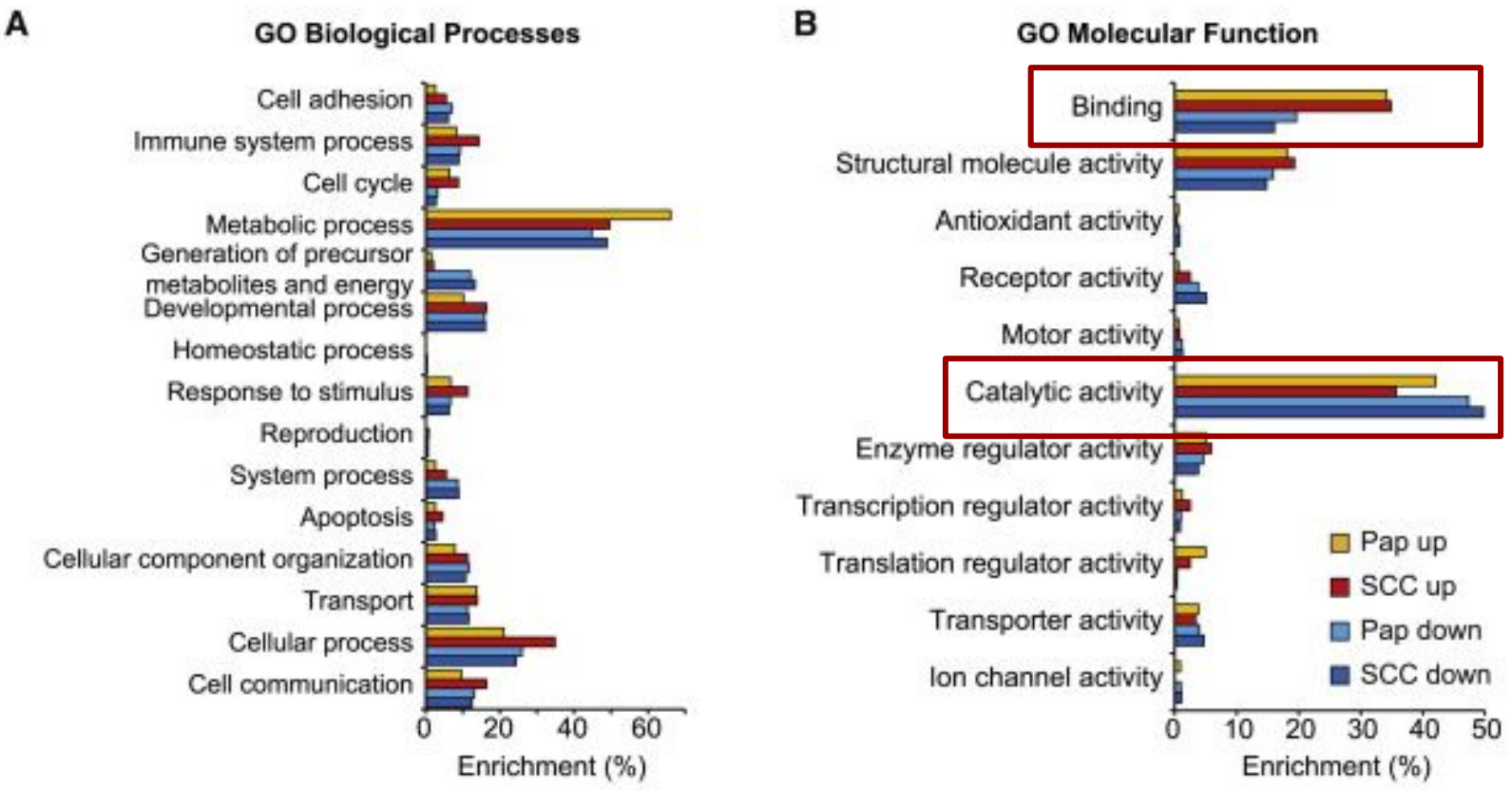


TPA, Pap, and SCC showed high similarity (average R2 of 0.9 for proteome and 0.8 for phosphoproteome)



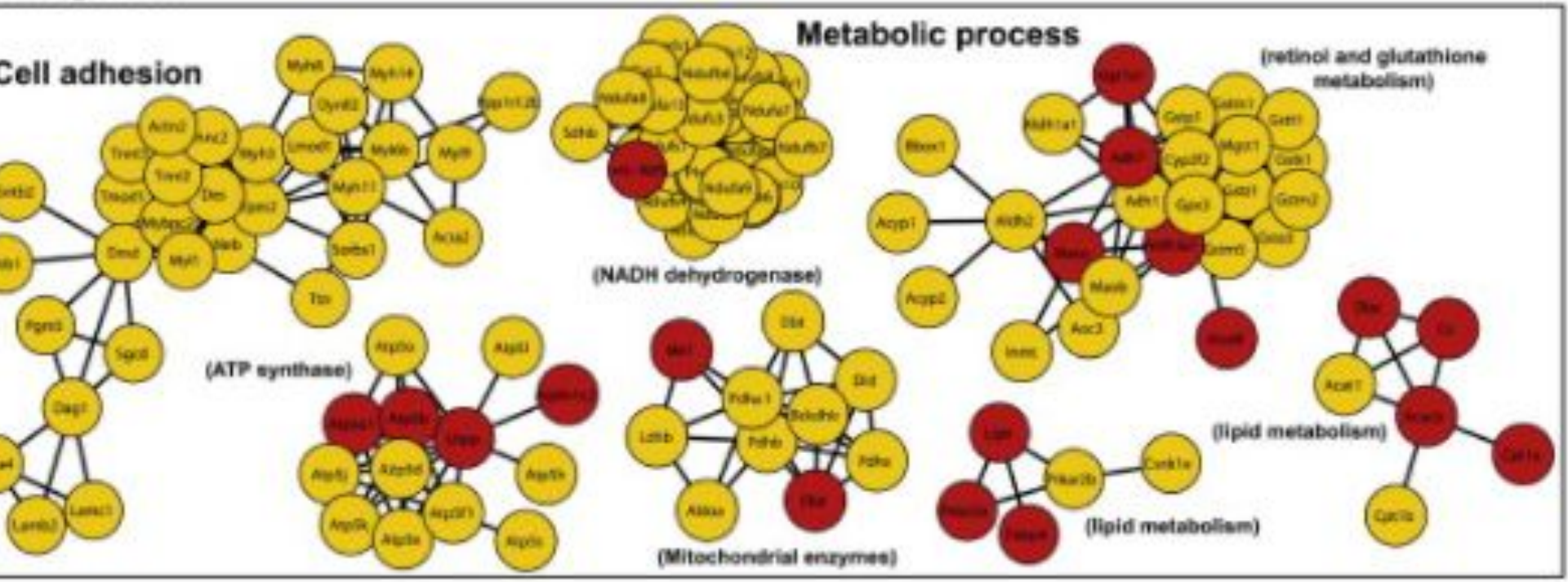
Correlation only .5 for the control skin Low correlation was attributed to the non-reproducible isolation of the samples that occurred in the skin samples alone

How did the proteomic study reinforce previous molecular mechanisms in tumorigenesis?

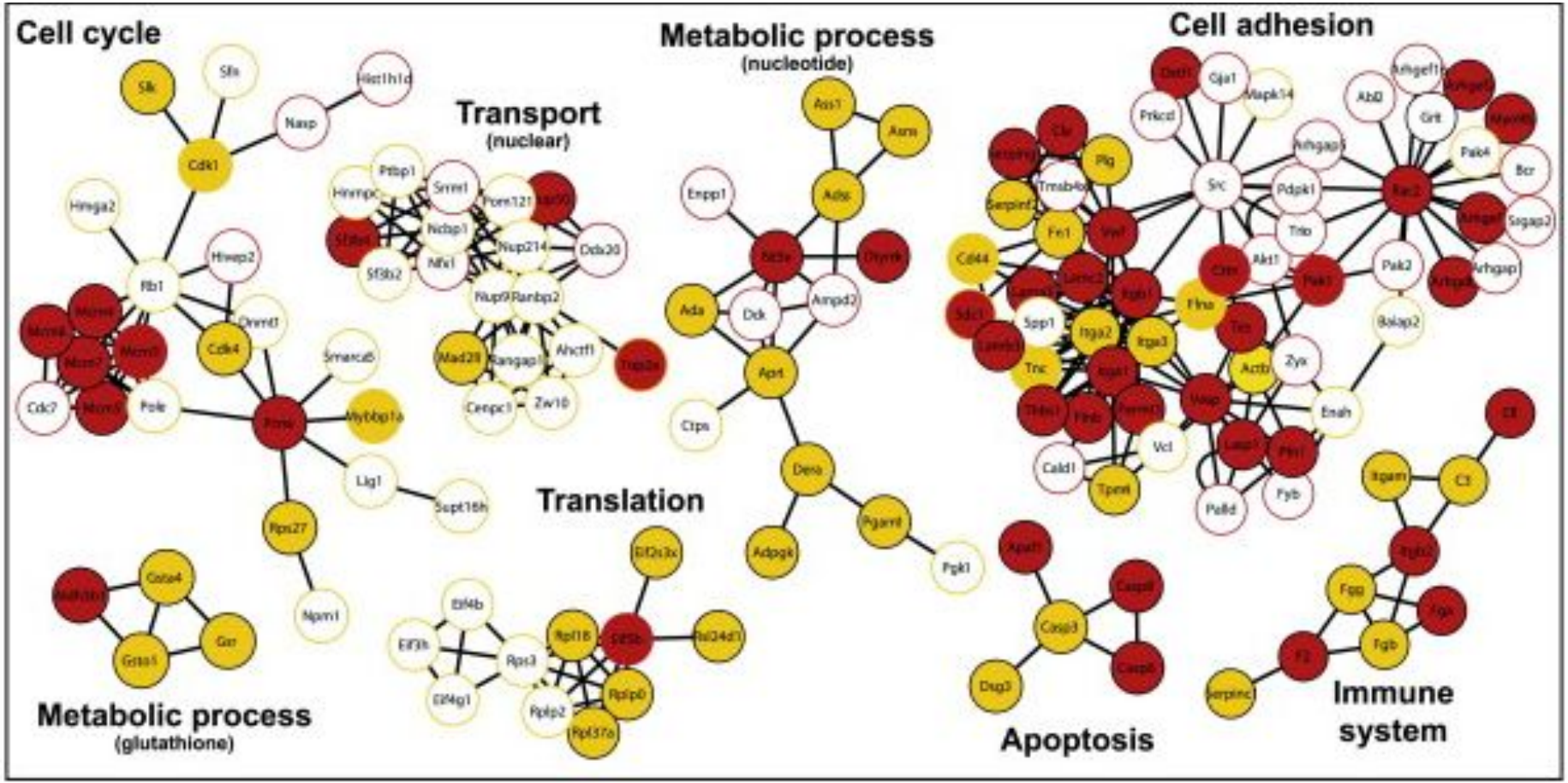

A
GO Biological Processes
Cell adhesion
Immune system process
Cell cycle
Metabolic process
Generation of precursor metabolites and energy
Developmental process
Homeostatic process
Response to stimulus
Reproduction
System process
Apoptosis
Cellular component organization
Transport
Cellular process
Cell communication
0
20
40
60
Enrichment (%)
B
GO Molecular Function
Binding
Structural molecule activity
Antioxidant activity
Receptor activity
Motor activity
Catalytic activity
Enzyme regulator activity
Transcription regulator activity
Translation regulator activity
Transporter activity
Ion channel activity
Pap up
SCC up
Pap down
SCC down
0
10
20
30
40
50
Enrichment (%)

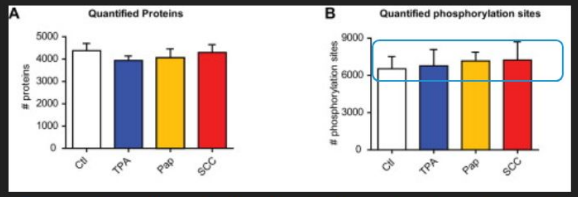
How do kinase activities look in the mouse tumors?

Downregulation

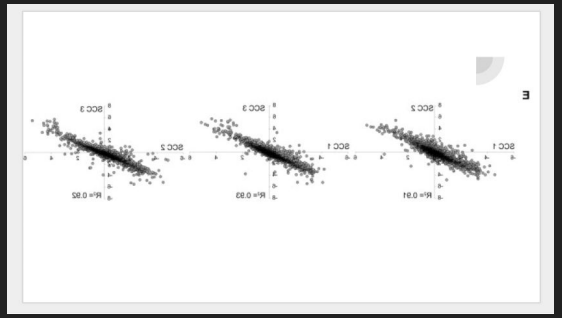


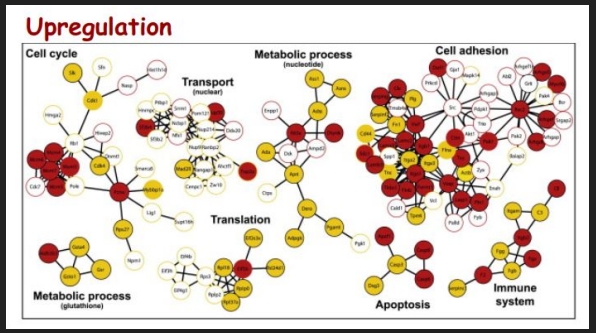
Upregulation





Increased number of phosphorylation sites found in each tissue sample vs control showing an increasing trend with later stages of skin carcinoma





SUMMARY

Proteomic analysis identified clear and distinct differences in protein expression levels between normal keratinocytes and tumor cells

Proteomic data strongly highlights PAK4-PKC/SRC subnetwork with cell adhesion

Spike In SILAC Technology helps extend the approach to other mouse models and human tumors

Experimental set-up provides advantages for proteomic proteomics quantification and interpretation